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Award Number: DAMD17-03-1-0403

TITLE: Mechanisms by Which Distinct ErbB Receptor Homo- and Heterodimers Reinitiate Proliferation in Growth-Arrested, Polarized, Epithelial Structures

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REPORT DATE: June 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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**REPORT DOCUMENTATION PAGE**Form Approved  
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<b>1. AGENCY USE ONLY</b> (Leave blank)		<b>2. REPORT DATE</b> June 2004	<b>3. REPORT TYPE AND DATES COVERED</b> Annual Summary (1 Jun 03-31 May 04)	
<b>4. TITLE AND SUBTITLE</b> Mechanisms by Which Distinct ErbB Receptor Homo- and Heterodimers Reinitiate Proliferation in Growth-Arrested, Polarized, Epithelial Structures			<b>5. FUNDING NUMBERS</b> DAMD17-03-1-0403	
<b>6. AUTHOR(S)</b> Bin Xiang, Ph.D.				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Cold Spring Harbor Laboratory Cold Spring Harbor, New York 11724  E-Mail: xiang@cshl.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b> Original contains color plates. All DTIC reproductions will be in black and white.				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited				<b>12b. DISTRIBUTION CODE</b>
<b>13. ABSTRACT (Maximum 200 Words)</b>  Using three-dimensional epithelial culture and inducible ErbB activation methods, we analyzed the ability of ErbB2/ErbB3 heterodimer to induce proliferation of epithelial cells in acini, and investigated the mechanism by which ErbB2 regulates cell cycle progression. Our data shown that ErbB2/ErbB3 heterodimer has stronger ability to initiate proliferation of growth-arrested cell in acini, meanwhile, to protect cells from apoptosis, compared to ErbB2 homodimer. Cyclin D1, D3 and E1 are required for normal morphogenesis of 3D acini, but only cyclin E1 is required for ErbB2-induced proliferation. ErbB2-overexpressed breast cancer cell lines or primary tumors have higher levels of cyclin E.				
<b>14. SUBJECT TERMS</b> ErbB, breast cancer, three-dimensional epithelial culture, cell cycle				<b>15. NUMBER OF PAGES</b> 14
				<b>16. PRICE CODE</b>
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

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### Introduction

It is estimated that one in eight women will develop breast cancer in their lifetime. ErbB oncogene family of receptor tyrosine kinase has been shown to be involved in breast cancer formation. Among its four members, ErbB2 overexpression is observed in 25-30% of breast cancers and correlated with poor clinical prognosis. ErbB2 is targeted for therapy using anti-ErbB2 antibody, Herceptin. Mammary epithelial cells express multiple ErbB receptors and at least 10 different growth factors as their ligands. Upon binding to their ligands, ErbB receptors form homodimer or heterodimer with other ErbB members. The dimerization activates ErbB receptor's tyrosine kinase activity and consequently induce phosphorylation of ErbB receptors which transfer the external signals into intracellular pathway, like activation of MAPK pathway, PI3K/Akt pathway. Evidence shown that ErbB2/ErbB3 heterodimer has the strongest mitogenic potential. To date, the mechanism by which ErbB homodimer and heterodimer controls breast epithelial cell proliferation is unclear. Also, there is limited evidence to uncover the mechanism of ErbB2-regulated cell cycle progress.

In our lab, we have chemical-induced dimerization of ErbB system which allows us to activate specific ErbB dimers independently on endogenous growth factors. This gives me an ideal tool to study the ErbB dimer's signaling. Also, we established Matrigel-based three-dimensional (3D) mammary epithelial cell culture system. The single cell can form acini-like structures on Matrigel which shares several characteristics of lobular acinar structures *in vivo*. People believe that 3D culture, comparing to the regular plastic dish culture (2D), takes one step closer to *in vivo* situation to investigate oncogenesis. I combine these two approaches to study ErbB oncogene's roles in transformation of breast epithelial cells.

## Body

### Training

In the period June 1, 2003 to May 31, 2004, I got solid trainings as follow: Matrigel based three-dimensional epithelial cell culture, ErbB signaling transduction and cell cycle analysis, making retroviruses, RNAi, mouse mammary gland transplant.

### Accomplishments

**Task 1:** a. Prepared retroviral stocks for ErbB1, 2, 3 and ErbB4 chimeras.

b. Generated MCF-10A cell lines stably expressing ErbB1, ErbB2, ErbB3, ErbB4, ErbB1 and ErbB2, ErbB1 and ErbB3, ErbB1 and ErbB4, ErbB2 and ErbB3, ErbB2 and ErbB4, or ErbB3 and ErbB4.

c. Tested the signal transduction induced by ErbB activation. ErbB1 or ErbB3 homodimer doesn't induce downstream signaling. ErbB2 homodimer induces activation of MAPK pathway. ErbB4 couldn't get phosphorylated by induction of dimerizer. Among the heterodimers, ErbB2/ErbB3 heterodimers (in ErbB2 ErbB3 co-overexpressed cells) strongly activates PI3K/Akt pathway compared to ErbB2 homodimer or ErbB1/ErbB2 heterodimer or ErbB1/ErbB3 heterodimer.

d. With 3D culture, I identified that ErbB2 homodimer, ErbB1/ErbB2 heterodimer, and ErbB2/ErbB3 heterodimer can initiate proliferation in MCF-10A acinar structures.

Difficulties: the construct of ErbB chimera only has FKBP domain which binds drugs like AP1510 to form homodimers. To study heterodimer, we tested FRB-containing constructs. The drug only induces weak heterodimers. We are engineering the constructs by putting Coumermycin-GyrB based drug binding domain instead of FKBP domain.

**Task 2:** a and c. I am doing microarray analyzing the gene profiles of MCF-10A cells in 2D and 3D system including identifying cell cycle genes induced by ErbB2 homodimer.

b. Identified that cyclin E is the major target of ErbB2 homodimer for initiating proliferation in 3D.

d. I am creating the shRNA library against more than 20 different polarity-related genes. I am going to screen the polarity genes hairpins with proliferation assays.

**Task 3:** a. Instead of generation of transgenic mice, I already set up the fat pad transplantation system. I am going to engineer the mouse primary epithelial cells or stem cells by retroviral infection and transplant them back to the mammary gland cleared fat pad.

b. I didn't start yet.



### Key Accomplishments

#### 1: ErbB2/ErbB3 heterodimer has stronger ability to transform breast epithelial cells in three-dimensional system compared to ErbB2/ErbB2 homodimer

The ErbB chimera contains extracellular and intracellular domain of NGFR, ErbB cytoplasmic domain, FKBP domain which is the dimerizer drug-binding domain, and HA tag (Muthuswamy et al., 1999). We use immortalized normal breast epithelial cell MCF-10A to study the transformation ability of ErbBs. Our lab already established ErbB2 homodimerization system by expressing ErbB2 chimera into MCF-10A stably. The previous data shown that dimerizer drug such as AP1510 could induced the dimerization of ErbB2 chimera and activated downstream signals as wildtype ErbB2 receptor. Also, ErbB2 homodimer could initiate the proliferation of growth-arrested MCF-10A in acini on Matrigel (Muthuswamy et al., 2001). Some publication suggested that ErbB2 is the preferred partner for heterodimer with other ErbBs and ErbB2/ErbB3 heterodimer has strongest mitogenic potential (Yarden and Sliwkowski, 2001). Since most of the phenotype were observed by either overexpression ErbB receptors or using breast cancer cell lines and fibroblast cells. Here, we tested if ErbB2/ErbB3 heterodimer has stronger transformation ability by using our ErbB activation inducible system in normal breast epithelial cells.

We used the MCF-10A stably expression ErbB2 chimera (ErbB2) as host cell and expressed ErbB3 chimera, and selected to get cells stably expression of both ErbB2 and ErbB3 (ErbB2.ErbB3). Since the ErbB2 or ErbB3 chimera has the same dimerizer-binding domain, application of AP1510 would result in formation of ErbB2/ErbB2 homodimer, ErbB3/ErbB3 homodimer, and ErbB2/ErbB3 heterodimer. ErbB3 is kinase dead receptor and ErbB3/ErbB3 homodimer would have no signal. The signals upon dimerizer stimulation in ErbB2.ErbB3 cells should be from ErbB2/ErbB2 homodimer and ErbB2/ErbB3 heterodimer. And, the ErbB2 levels are the same in both ErbB2 cells and ErbB2.ErbB3 cells. Then, the different phenotype in ErbB2.ErbB3 cells, compared to ErbB2 cells, should be from ErbB2/ErbB3 heterodimer.

##### 1.1 The signals induced by dimerizer

To measure the activation of ErbB chimera, we detected the phosphorylation of ErbBs induced by dimerizer under plastic dishes culture (2D) condition. The ErbB2 and ErbB3 levels were shown in Fig 1.1a. To detect if ErbB chimeras can be activated, as shown in Fig 1.1b, cells were incubated with 1  $\mu$ M dimerizer, AP1510, the phosphorylation of ErbB2 or ErbB3 were measured by Western blot. The downstream signals such as phosphorylated-ERK, phosphorylated-Akt were tested by Western blot (Fig 1.1c). The results indicated that, dimerizer can induce the phosphorylation of ErbB chimeras, ErbB2 homodimer and ErbB2/ErbB3 heterodimer can activate downstream signals as the wild type ErbBs. Specially, ErbB2/ErbB3 heterodimer can induce strong activation of Akt.

##### 1.2 Morphogenesis of 3D acini on Matrigel

We observed the 3D morphogenesis of ErbB2 and ErbB2.ErbB3 cells. As shown in Fig 1.2a, both cell lines gave the similar morphology of 3D acini during the development of acinar structures. One thing should point out is in ErbB2.ErbB3 acini at day 14, there are little higher background of multi-acinar structures compared to ErbB2. After activation by dimers for 4 days, some acini formed multi-acinar structure. We counted the number of multi-acini among at least 1000 acini. There are more multi-acini in ErbB2.ErbB3 (40-45%) compared to ErbB2 acini (25-30%) (Fig 1.2b).

##### 1.3 Regulation of apoptosis in acini

We looked at the difference of the ability of homodimer and heterodimer to regulate apoptosis. We activated the dimers at day 6 by adding dimerizer to the acini medium and fixed and stained the acini structures at day 8 with anti-cleaved caspase 3 antibody. Confocal images were took and the cleaved caspase 3 positive acini were counted among at least 500 acini. As shown in Fig 1.3, the central cells underwent apoptosis at day 8, the stimulation of dimer reduced the cleaved caspase 3 staining on the central cells both in ErbB2 and ErbB2.ErbB3 acini. But the cleaved caspase 3 positive acini was much lower in ErbB2.ErbB3 acini than ErbB2 acini which implicated that ErbB2/ErbB3 heterodimer has higher ability to negatively regulate apoptosis.

##### 1.4 Ability to induce proliferation by staining Ki-67

We, here, compared the ability of ErbB2 homodimer and ErbB2/ErbB3 heterodimer to initiate proliferation. We stimulated the acini at day 14 with dimerizer for 2 days and fixed and stained the acini with antibody against Ki-67. The level of Ki-67 in acini without dimerizer treatment was low, but increased



dramatically after stimulation with dimerizer (Fig 1.4a). We quantitated the Ki-67 positive acini among at least 1000 acini (Fig 1.4b). Ki-67 positive acinus means there are at least 6 Ki-67 positive cells in one acinus. We also detected the cdk2 kinase activity induced by different dimers. As shown in Fig 1.4c, ErbB2/ErbB3 heterodimer induces higher kinase activity of cdk2, about 4 folds more than ErbB2 homodimer-induced. The results indicated that ErbB2/ErbB3 heterodimer has stronger ability to initiate proliferation in growth-arrested acini compared to ErbB2 homodimer.

## **2: Cyclin E is Required for ErbB2-induced Disruption of 3D Mammary Epithelial Acini**

To better understand the signaling of ErbB dimers during regulation of proliferation in 3D acini, we choose ErbB2 homodimer to study this question. We knew that ErbB2 homodimer can initiate proliferation of growth-arrested breast epithelial acini (Muthuswamy et al., 2001). Plenty of studies using fibroblast cells or cancerous cell lines cultured on plastic dishes gave us some ideas about how ErbB2 regulates cell cycle (Neve et al., 2000; Lee et al., 2000; Lane et al., 2000; Lenferink et al., 2001). But, it is still unclear how ErbB2 controls epithelial cells proliferation during breast tumor formation. For example, it implicated that ErbB2 control the cell cycle through cyclin D1. However, studies using primary human breast tumors demonstrate that ErbB2-overexpression tumor doesn't express high level of cyclin D1 (Yang et al., 2004). Here we demonstrate that while both D-type cyclins and E-type cyclins play critical roles during normal morphogenesis of breast epithelial cells, ErbB2-induced transformation of mammary epithelial acini requires cyclin E1 but not cyclin D1 or D3. In addition, ErbB2 expression correlates with cyclin E overexpression in primary breast cancers and ErbB2 overexpressing breast cancer derived cell lines suggesting that cyclin E plays an important role in ErbB2-induced G1/S progression both in vitro and in vivo.

### **2.1 Mammary epithelial cells arrest at G1 phase during 3D morphogenesis**

In 3D condition, the cells undergo several rounds of cell division during the initial period (until Day10) of morphogenesis and subsequently undergo a proliferation-arrest and remain arrested for an extended period of time. In order to investigate manner in which the G1 regulators are affected during morphogenesis, we lysed the acini at different stages of development (as described in Fig 2.1) and monitored the expression of G1 phase regulators. Consistent with a G1 arrest, we observed a decrease in the levels of p-Rb and was undetectable after Day10 (Figure 2.1b). Concomitantly, we observed an increase in the expression of a CDK inhibitor p27kip1. Among the cyclins, cyclin D3 was downregulated to undetectable levels after Day8, and cyclin E1, D1, and D2 show a slight decrease (Fig 2.1c). Taken together these observations suggest that the breast epithelial cells undergo a G1 phase arrest during 3D morphogenesis and involve changes in specific cyclins and a CDK inhibitor (p27kip1).

### **2.2 D Type and E Type cyclins are critical for normal morphogenesis**

We silenced the expression of Cyclin D1, D3 and E1 by RNA interference (RNAi). Transfection of small interfering RNAs (siRNA) pools (SMARTpool<sup>TM</sup>) against the cyclins downregulated expression of the cyclins being targeted (Fig 2.2a). Cells transfected with siRNAs were subject to 3D morphogenesis and the development of structures were monitored during the first 4 days of culture (Fig 2.2b). Phase images were recorded and the size distribution of approximately 1000 structures was estimated. Based on the size distribution observed in parental MCF-10A cells, acini that were smaller than 15  $\mu$ m in diameter were considered abnormal while larger acini were considered normal. Inhibition of Cyclin D1, D3 or E1 expression resulted in more than two fold decrease in the number of normal size acini (Fig 2.2c) suggesting that all three cyclins play critical roles during acini morphogenesis.

### **2.3 ErbB2 activates Cyclin E/Cdk2 but not Cyclin D/Cdk4 in 3D acini**

Activation of ErbB2 re-initiates proliferation in proliferation-arrested acini as monitored by changes in the expression of Ki67, a marker for proliferating cells (Fig 1.4). In addition, activation of ErbB2 induced 2-3 fold increase in the expression of Proliferating Cell Nuclear Antigen (data not shown). Activation of ErbB2 induced an increase in the levels phosphorylated form of Rb and an increase in the levels of cyclin D3 and cyclin E1 (Fig 2.3a). However, we did not observe any significant changes in the levels of cyclin D1 (Fig 2.3a). In addition, the levels of the CDK inhibitor, p27kip1, decreased upon activation of ErbB2 (Fig 2.3a). In order to determine whether changes in expression of cyclins and CDK inhibitor affected CDK activities, we investigated changes in CDK2 and CDK4 kinase activity in acini. Activation of ErbB2 induced a significant increase in CDK2 kinase activity (Fig 2.3bi) but no detectable



change in CDK4 activity (Fig 2.3bii). In order to determine whether activation of ErbB2 in cells grown on 3D matrices differ from activation in 2D in its ability to induce cyclin D1, we activated ErbB2 in MCF-10A cells grown in 2D plastic dishes. Activation of ErbB2 in 2D induced a modest (1.5 fold) increase in the levels of cyclin D1 and increase in CDK4 kinase activity (Fig 2.3c) suggesting that the ability of ErbB2 to regulate cyclin D1/CDK4 may differ between 2D and 3D.

#### **2.4 ErbB2-induced S phase entry requires Cyclin E1 but not D1 or D3**

To determine the role played by Cyclins D1, D3 or E1 in ErbB2-induced proliferation, expression of various cyclins were individually repressed in 10A.ErbB2 cells using siRNA. Activation of ErbB2 in the absence of Cyclin D1 or Cyclin D3 did not significantly (p value for D1=0.12 and for D3=0.16) affect the ability of ErbB2 to induce S-phase entry as monitored by the ability of cells to incorporate BrdU (Fig 2.4). Whereas, activation of ErbB2 in the absence of Cyclin E1 significantly impaired the ability of ErbB2 to induce S-phase entry (p value =0.0007). These observations suggest that although ErbB2 induces expression of Cyclin D3 and Cyclin E1, only E1 is required for ErbB2-induced proliferation.

#### **2.5 ErbB2-induced formation of multi-acinar structures requires Cyclin E1**

We have previously shown that activation of ErbB2 in proliferation arrested 3D acini induces proliferation and formation of multiacinar structures. ErbB2 was activated in control or siRNA transfected cells, phase images were recorded (Fig 2.6a). Percentage of multiacinar structures was determined by counting about 1000 structures. Activation of ErbB2 resulted in 30% multiacinar structures, loss of Cyclin D1 or D3 did not significantly affect the percentage of multiacinar structures. By contrast, loss of E1 expression significantly inhibited the ability of ErbB2 to induce multiacinar structures (Fig 2.6 a, b) suggesting Cyclin E1 plays a critical role in ErbB2-induced disruption of 3D acini.

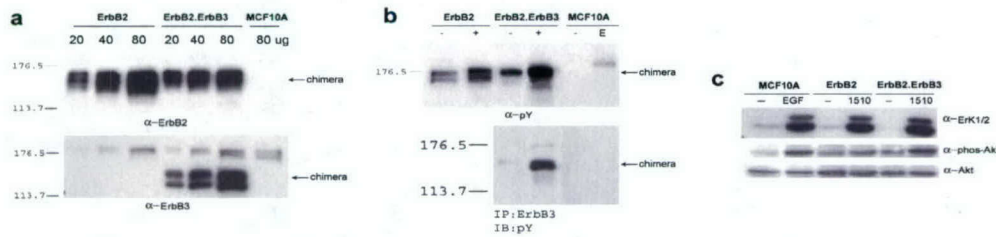
#### **2.6 Expression of Cyclin E in ErbB2-overexpressing breast cancer cell lines and primary tumor tissues**

To explore whether there is a relationship between ErbB2 overexpression and high level of Cyclin E expression, we analyzed a panel of human breast cancer cell lines and breast tumor tissues. MCF-10A cells were used as a control (Fig 2.6). Among the breast cancer cell lines tested, both ErbB2 overexpressing cell lines (ZR-75-1 and SK-BR-3) expressed high levels of Cyclin E1 while only ZR-75-1 expressed higher levels of Cyclin D3 suggesting a relationship between ErbB2 overexpression and Cyclin E overexpression. None of the cell lines expressed significantly higher levels of Cyclin D1 (Fig 2.6a).

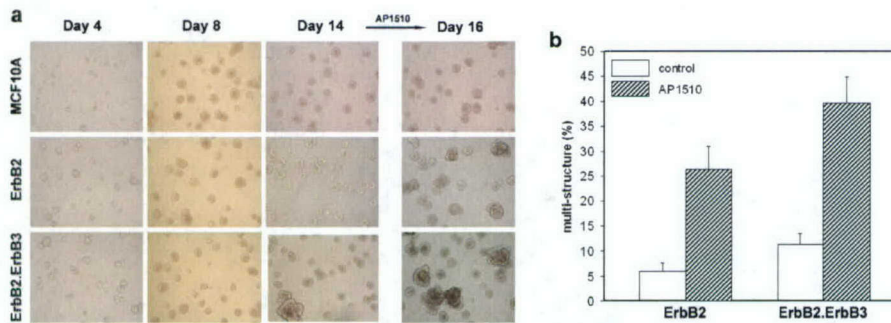
To determine if ErbB2 overexpression correlates with high levels of Cyclin E1 in primary breast tumors we analyzed nine tumor tissues (Fig 2.6b). Of the five ErbB2-overexpression tumors, 4 tumors had higher level of Cyclin E1. We note that 4/5 ErbB2 positive tumors had higher levels of Cyclin D3 with three tumors expressing higher levels of both Cyclin D3 and E1. Taken together our results suggest Cyclin E1 plays a critical role in ErbB2 induced proliferation in vitro and in vivo.



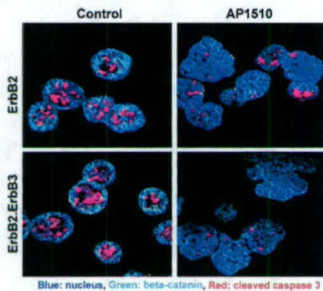
## Figures



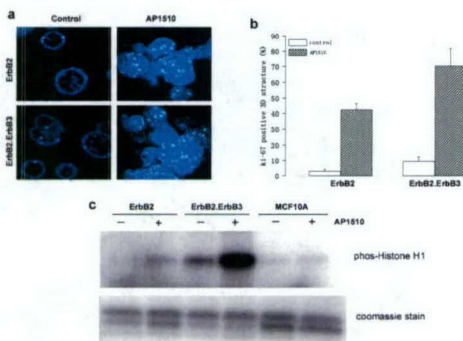
**Fig 1.1: The cell lines and signaling.** MCF10A cells were infected with retroviruses carrying ErbB2 chimera (ErbB2), ErbB2 cells were infected with retroviruses carrying ErbB3 chimera (ErbB2.ErbB3). **a**, the receptors level was measured with antibody against ErbB2 or ErbB3 by Western blot. **b**, receptor phosphorylation was detected after stimulated with 1  $\mu$ M AP1510 (+) or EGF (E) for 15 min with antibody against phosphor-tyrosine (pY). **c**, downstream signals, Erk1/2 and Akt, were detected by Western blot after stimulated with AP1510 (1510) or EGF for 15 min.



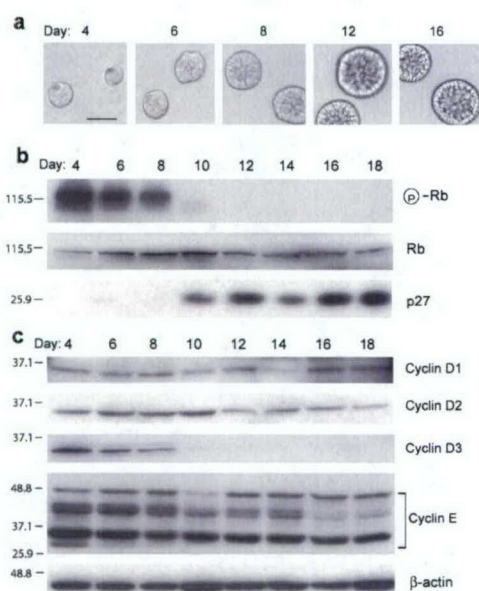
**Fig 1.2: Morphogenesis of ErbB2 and ErbB2.ErbB3 acini.** **a**, Stable cell lines were subjected to Matrigel, the morphology was pictured with microscopy at different stage. Day 14 old acini were incubated with 1  $\mu$ M AP1510 for 2 days, the multi-acinar structures were counted among at least 1000 acini and the percentage was shown in **b**.



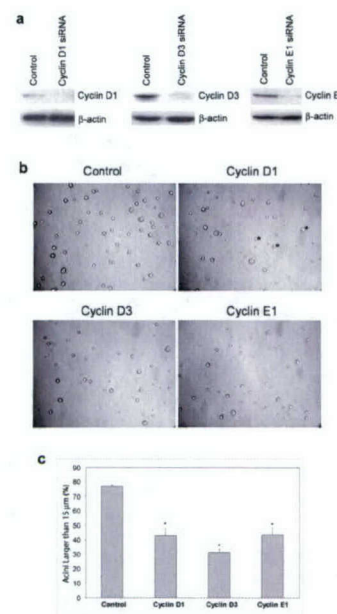
**Fig 1.3: Regulation of apoptosis in acini.** Day 6 old acini were stimulated with 1  $\mu$ M AP1510 or not for 2 days, at day 8, the structures were fixed and stained with antibody against cleaved caspase 3 (red) and beta-catenin (green). The nucleus were stained by Topro-3 (blue).



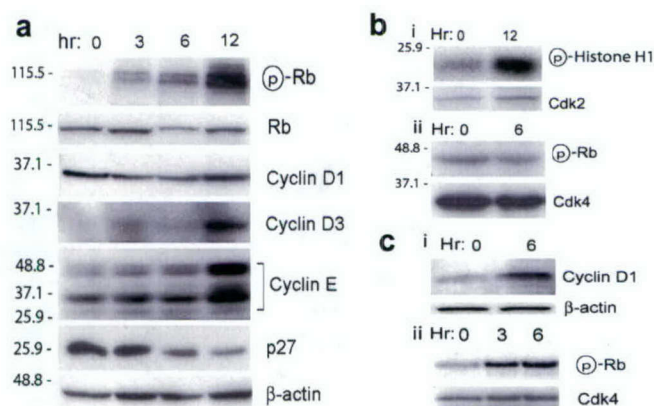
**Fig 1.4: Proliferation in acini induced by different dimers.** **a**, Day 14 old acini were stimulated with 1  $\mu$ M AP1510 for 2 days, and day 16 old acini were fixed and stained with antibody against Ki-67 (green). The nucleus were stained with Topro-3 (blue). Ki-67 positive acini were quantified among at least 500 acini and percentage was shown in **b**. **c**, cdk2 was immunoprecipitated from ErbB2, ErbB2.ErbB3, or MCF10A acini lysate, kinase activity assay in vitro was performed using Histone H1 as substrate.



**Fig 2.1: G1 regulators during morphogenesis of mammary epithelial 3D acini.** **a**, MCF-10A cells were cultured on Matrigel, and images that represent different stages of morphogenesis are shown. The scale bar, 50  $\mu$ m. **b,c**: day 4 to day 18 old acini structures were lysed and levels of phosphorylated-pRb, Rb, p27, cyclinD1, cyclin D2, cyclin D3, cyclin E, or cyclin E2 were assessed. Blot was reprobed with anti- $\beta$ -actin to serve as a loading control.



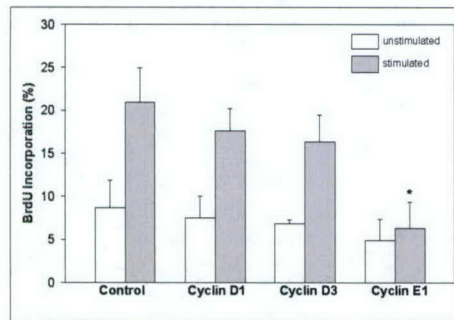
**Fig 2.2: G1 cyclins are required for morphogenesis of 3D acini.** **a**: SMARTpool<sup>TM</sup> siRNAs against cyclin D1, cyclin D3 or cyclin E1 were transfected into MCF-10A cells and the cyclins levels were detected by immunoblot with indicated antibodies. **b**: siRNA-transfected cells were cultured on Matrigel, and phase images were recorded at day 4. **c**: Percentage of normal size of acini ( $\geq 15 \mu$ m) (indicated as asterisk in b) was quantified from at least 1000 acini for each experimental group. The results are average from two separating experiments. Error bars represent s.d. \*,  $p < 0.001$  compared with the control group



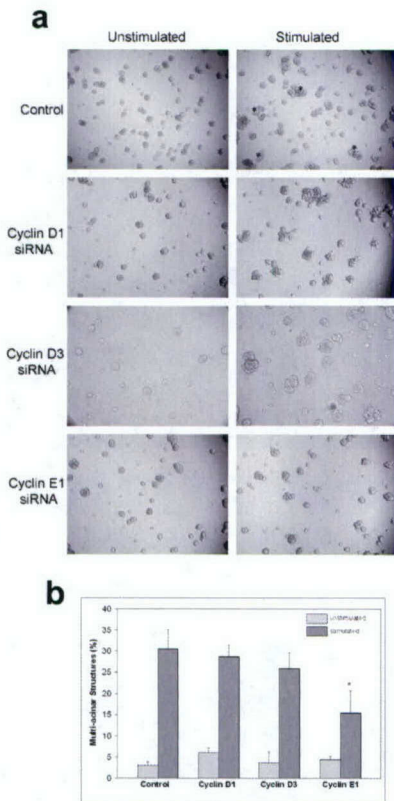
**Fig 2.3: ErbB2 activates Cyclin E/cdk2 but not Cyclin D/cdk4 in 3D acini.** **a**: ErbB2 cells were cultured in 3D culture system, and the day 14 old acinar structures were stimulated with 1  $\mu$ M AP1510 for 0, 3, 6, 12, or 24 hours and lysed. The phosphorylated pRb, pRb, cyclin D1, D3, E, or p27 was assessed by Western blot analysis with indicated antibodies. Blot was reprobed with anti- $\beta$ -actin to serve as a loading control. **b**: ErbB2 acinar structures were stimulated with 1  $\mu$ M dimerizer with indicated time and lysate was prepared. The cdk2- (i) or cdk4- (ii) kinase activities were measured using Histone H1 or Rb as a substrate, respectively. Gels were transferred to PVDF membrane. The membranes were exposed with films and then blotted with anti-cdk2 or cdk4 antibodies. **c**: ErbB2 cells were cultured on plastic dishes and stimulated by



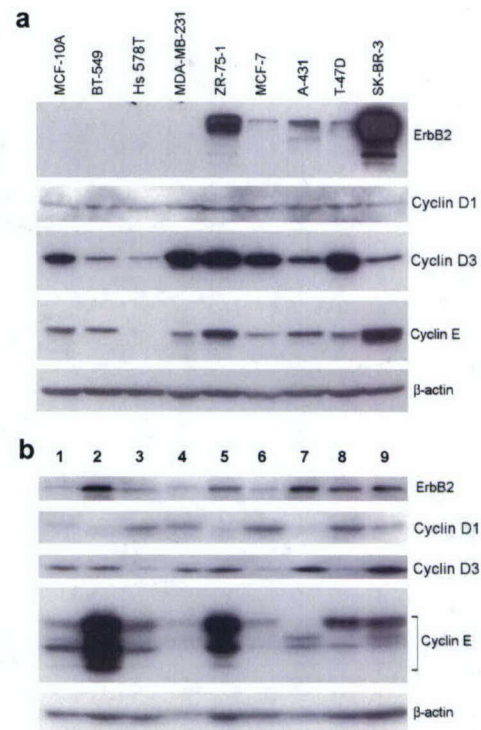
dimerizer with indicated time. Cyclin D1 level was detected (i) by Western blot analysis. Cdk4 kinase activity was measured using Rb as a substrate.



**Fig 2.4: Cyclin E is required for ErbB2-induced S phase entry.** ErbB2 cells were, cultured on glass coverslips, transfected with siRNAs against cyclinD1, D3, or E1 and stimulated with or without 1  $\mu$ M dimerizer. BrdU incorporation assay was conducted. Percentage of BrdU-positive nucleus were quantified from at least 1500 nucleus for each experiment. The results are average from three separating experiments. Error bars represent s.d. \*,  $p < 0.001$  compared with stimulated control experiment.



**Fig 2.5: Cyclin E is required for ErbB2-induced multi-acinar structure formation in 3D.** ErbB2 cells transfected with siRNAs against cyclin D1, D3, or E1 were cultured in 3D system. Day 4 old structures were stimulated with or without 1  $\mu$ M dimerizer for 3 days. **a:** Day 7 old structures were pictured. Asterisk (\*) indicates the multi-acinar structure. **b:** Percentage of multi-acinar structures was quantified from at least 1000 structures for each experiment. Error bars represent s.d. \*,  $p < 0.01$  compared to stimulated control experiment.



**Fig 2.6: Expression of cyclins in breast cancer cell lines and breast tumor tissues.** **a:** Human breast cancer cell lines BT-549, Hs 578T, MDA-MB-231, ZR-75-1, MCF-7, T-47D, SK-BR-3, epidermoid carcinoma cell line A-431, and MCF-10A cells were cultured on plastic dishes. **b:** Human breast tumor tissues were lysed. Lysates were analyzed by SDS-PAGE. ErbB2, cyclin D1, cyclin D3, and cyclin E expression were measured by Western blot analysis with indicated antibodies. Blot was reprobbed with anti- $\beta$ -actin to serve as a loading control.

### Reportable Outcomes

Abstract: Non-transformed breast epithelial cells undergo morphogenesis that results in formation of proliferation-arrested acini-like structures when plated on 3D matrix. Here we demonstrate that the levels of the cyclin E1 and D3, and the levels of phosphorylated Rb decreased during morphogenesis. Whereas, the levels of the cell cycle inhibitor p27 increases during morphogenesis suggesting that the mammary epithelial cells arrest at G1 phase of the cell cycle during morphogenesis. RNA interference mediated decrease in the levels of Cyclin D1, Cyclin D3 or Cyclin E1 inhibit normal morphogenesis suggesting that G1 cyclins play critical role during normal breast epithelial morphogenesis. Activation of ErbB2 in fully formed 3D acini results in an increase in the levels of Cyclins E1, D3, but not D1, and an increase in the CDK2 activity, but not cdk4, with a concomitant increase in Rb phosphorylation. RNA-interference mediated down regulation of Cyclin E1, but not cyclin D1 or D3, inhibited ErbB2-induced proliferation and disruption of 3D acini suggesting that ErbB2 specifically requires Cyclin E1 for deregulation of proliferation control in 3D acini. Expression of RasV12 resulted in an increase in the levels of Cyclin D1, D3 and E1 suggesting the ErbB2 and RasV12 differ in their ability to regulate G1 Cyclins. In addition, overexpression of ErbB2 correlated with increased expression of Cyclin E1 but not D1 or D3 in both breast cancer derived cell lines and primary breast tumors suggesting that Cyclin E1 plays a critical role in ErbB2-induced G1 progression both *in vitro* and *in vivo*. Taken together these observations suggest that oncogenes differentially regulate G1 cyclins in 3D acini and that oncogene-mediated regulation of cell cycle in 3D acini may allow us to identify relationship that in occur in primary breast cancers.



### **Conclusion and Future Plans**

Taken together, our data show that ErbB2/ErbB3 heterodimer has stronger ability to induce proliferation of breast epithelial acini. Cyclin E, but not cyclin D1, D3, is the critical downstream mediator of ErbB2 signaling to control the proliferation in 3D acini.

For future plans, in task 1, I will test the GyrB-based construct in MCF-10A cells and establish more heterodimer models to study signals of different homo- and heterodimers in proliferation in 3D. In task 2, I will finish the microarray analysis and compare the gene patterns in 2D and 3D system. At the same time, I will screen the polarity gene short hairpins to figure out which polarity gene is involved in proliferation induced by ErbB2 homodimer. For task 3, I will continue the mouse fat pad transplant project and analyze different combination of ErbB2 and oncogenes or tumor suppressor genes to understand the mechanism of ErbB2-induced the mammary tumor formation.

### Reference

- Lane, H. A., Beuvink, I., Motoyama, A. B., Daly, J. M., Neve, R. M., and Hynes, N. E. (2000). ErbB2 potentiates breast tumor proliferation through modulation of p27(Kip1)-Cdk2 complex formation: receptor overexpression does not determine growth dependency. *Mol Cell Biol* 20, 3210-3223.
- Lee, R. J., Albanese, C., Fu, M., D'Amico, M., Lin, B., Watanabe, G., Haines, G. K., 3rd, Siegel, P. M., Hung, M. C., Yarden, Y., et al. (2000). Cyclin D1 is required for transformation by activated Neu and is induced through an E2F-dependent signaling pathway. *Mol Cell Biol* 20, 672-683.
- Lenferink, A. E., Busse, D., Flanagan, W. M., Yakes, F. M., and Arteaga, C. L. (2001). ErbB2/neu kinase modulates cellular p27(Kip1) and cyclin D1 through multiple signaling pathways. *Cancer Res* 61, 6583-6591.
- Muthuswamy, S. K., Gilman, M., and Brugge, J. S. (1999). Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. *Mol Cell Biol* 19, 6845-6857.
- Muthuswamy, S. K., Li, D., Lelievre, S., Bissell, M. J., and Brugge, J. S. (2001). ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. *Nat Cell Biol* 3, 785-792.
- Neve, R. M., Sutterluty, H., Pullen, N., Lane, H. A., Daly, J. M., Krek, W., and Hynes, N. E. (2000). Effects of oncogenic ErbB2 on G1 cell cycle regulators in breast tumour cells. *Oncogene* 19, 1647-1656.
- Yang, C., Ionescu-Tiba, V., Burns, K., Gadd, M., Zukerberg, L., Louis, D. N., Sgroi, D., and Schmidt, E. V. (2004). The role of the cyclin D1-dependent kinases in ErbB2-mediated breast cancer. *Am J Pathol* 164, 1031-1038.
- Yarden, Y., and Sliwkowski, M. X. (2001). Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2, 127-137.